STRUCTURAL MODIFICATION OF POLYSACCHARIDES: A BIOCHEMICAL-GENETIC APPROACH

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ABSTRACT

Polysaccharides have a wide and expanding range of industrial and biomedical applications. An industry trend is underway towards the increased use of bacteria to produce polysaccharides. Long term goals of this work are the adaptation and enhancement of saccharide properties for electronic and optic applications. In this report we illustrate the application of enzyme-bearing bacteriophage on strains of the enteric bacterium *Klebsiella pneumoniae*, which produces a polysaccharide with the relatively rare rheological property of drag-reduction. This has resulted in the production of new polysaccharides with enhanced rheological properties. Our laboratory is developing techniques for processing and structurally modifying bacterial polysaccharides and oligosaccharides which comprise their basic polymeric repeat units. Our research has focused on bacteriophage which produce specific polysaccharide degrading enzymes. This has lead to the development of enzymes generated by bacteriophage as tools for polysaccharide modification and purification. These enzymes have been used to efficiently convert the native material to uniform-sized high molecular weight polymers, or alternatively into high-purity oligosaccharides. Enzyme-bearing bacteriophage also serve as genetic selection tools for bacteria that produce new families of polysaccharides with modified structures.

INTRODUCTION

Polysaccharides are a ubiquitous, integral, and often invisible part of the manufacture and production of a very broad spectrum of products. Traditional uses of these materials include, but are not limited to, food additives, pharmaceutical chemicals, oil well drilling additives, industrial coatings, industrial cleaners, explosives, paint additives, paper chemicals, printing chemicals, photographic chemicals, textile chemicals, and additives for ceramics and metals[1]. Recent industrial applications of polysaccharides include drag-reducing agents for ships and water jet-cutting applications, photo-resist applications in the manufacture of integrated circuits, and the synthesis of new composite materials. Promising biomedical applications of polysaccharide themselves include a role in drug delivery and wound treatment[2].

Synthesis of new oligosaccharides with biological activity is an area of intense and growing interest. The discovery of the important and diverse role of saccharides in biological processes has lead to an increased demand for practical routes to gram scale quantities of saccharide-based compounds. Considerable progress has been made towards enzymatic approaches to oligosaccharide synthesis by a number of ingenious methods. Despite efforts in towards general methodologies, saccharide synthesis has been one of the most challenging areas of biochemistry[3]. The wide diversity of oligosaccharides produced by enzymatic polysaccharide degradation to its basic repeat unit provides a potential source of starting material for glycosylated pharmaceutical agents.

Hundreds of naturally occurring bacterial polysaccharides provide an untapped resource of chemically, structurally and functionally diverse biopolymers. Although polysaccharides have traditionally been derived from

plant and animal origin, the industry trend has been toward replacing these sources with microbially produced polymers. This is due to: (a) economic concerns, such as supply reliability and long term cost control, (b) quality control issues, such as product purity, and (c) application issues, which require consistency and specificity of the chemical and physical properties of the polysaccharide.

Despite these advantages, there are still technological problems which limit the production and isolation of polysaccharides from bacteria. Microbial bioreactors that produce polysaccharides generate a viscous fermentation liqueur that interferes with oxygen transfer, which in turn, limits microbial growth, and ultimately reduces polymer yield[4]. In addition, separating large amounts of polysaccharide from cells by conventional centrifugation is difficult because the polysaccharide is attached to the bacterial cell surface.

Although not impossible, the ability to produce and isolate new polysaccharides with modified structures from bacteria by conventional microbiological methods requires the following tedious approach. Strains producing potentially useful materials are isolated from the wild and converted to high yield strains by mutagenesis. This has been accomplished for capsular polysaccharides in a wide range of bacterial species[5,6,7]. Isolation of new polysaccharide structures requires extremely large scale screening of bacterial populations to isolate rare mutants with structurally-modified polysaccharides. The rarity of structural variants is due to the fact that polysaccharides are the end-product of complex multi-step enzymatic biosynthetic pathways. As such they are not readily amenable to modification by recombinant DNA technology. Typically, mutations, which result in the production of new structures, are too rare for the conventional screening approach to the problem to be viable.

Our laboratory has developed (and is in the process of refining) new and generally applicable techniques to produce and isolate high yields of structurally-modified polysaccharides and polysaccharases. We have utilized the interaction between bacteriophage and bacteria for the purification, processing and genetic modification of bacterial polysaccharides and their basic oligosaccharide repeat units. Bacteriophage, or simply phage, are virus like particles that infect and kill bacteria. The bacteriophage we have chosen to study distinguish themselves by producing a polysaccharide degrading enzyme, endoglycanase, which is specific for the polymer produced by the bacterial strain they infect. As a result, the bacterial polysaccharide capsule that would otherwise act as a physical barrier to phage infection is disrupted, permitting infection and resulting in cell death. Understanding the details of this process has lead to the development of a general set of biochemical and genetic techniques for the manipulation of polysaccharides. These techniques can be used to process and produce naturally occurring materials with desirable physical, mechanical, and biological properties.

APPROACH

The basic bacteriophage-based strategy for adapting bacteria for polysaccharide production is as follows. A strain producing a naturally occurring material with the property of interest is identified. If necessary, established mutagenesis techniques are used to produce a high yield polymer producing strain. Bacteriophage enzymes can be used to detach polymer product from the cells which produce them. Cells are then readily removed by a short low speed centrifugation leaving the polymer produced in solution. If desired, the material can be further processed to a high molecular weight form (greater than 500,000) by partial digestion of the material with purified bacteriophage enzyme. This yields material suitable for thin film fabrication. Alternatively, the material may be digested to its basic repeat oligomeric structure, with yields in excess of 50% and of high stereospecific purity due to the nature of the synthesis. If one seeks to alter the property of the native polymer, a population of bacterial cells is exposed to bacteriophage bearing endopolysaccharase specific to the native capsular polysaccharide. Those bacteria which are resistant to the phage often survive by virtue of producing a structurally modified capsular polysaccharide with reduced susceptibility to the bacteriophage enzyme.

The process we have developed to produce and isolate bacterial-derived polysaccharides can be illustrated by our search for polymers with "drag-reducing" properties.

The phenomenon of drag-reduction by suppression of turbulent flow in pipes has been studied for over forty years. Recently, this poorly understood non-newtonian phenomenon has been linked to the extensional viscosity of the active polymer[8]. These polymers have the ability to suppress turbulent flow at Reynolds numbers above the

transition point[9]. Several researchers have identified polysaccharides as polymers that have drag-reducing properties[10,11]. In fact, xanthan gum, a common industrial polysaccharide of bacterial origin is recognized as among one of the most effective drag-reducing polymers on a weight/ppm basis.

To test our process of producing and isolating bacterially-derived polysaccharides we have obtained a copy of the World Health Organization's *Klebsiella pneumoniae* serotypes, which include 79 closely related strains that produce capsular polysaccharides of known chemical structure. These strains were screened for production of drag-reducing polymers.

Several *K. pneumoniae* strains were found to produce drag-reducing material strain K63 produced one of the most effective polysaccharides. The native K63 polysaccharide, with its basic repeat unit of [galactose-acetylated galacturonic acid-fucose]$_n$, was found to be approximately twice as effective as a drag-reducer as xanthan gum[12]. The polymer also showed a high degree of resistance to mechanical shearing, as measured by repeated passage through a turbulent flow rheometer. Without bacteriophage-enzyme treatment it was impossible to effectively separate the polysaccharide from cells, even at centrifugation speeds of 50,000 x g for 4 hours. Brief bacteriophage-enzyme treatment, however, permitted rapid cell polymer separation of high molecular weight material (approximately 3,500,000), after 20 minutes of centrifugation at 6,000 x g. Furthermore, the ability to remove polysaccharide from the cell surface lead to an eighty percent increase in polymer recovery.

As depicted in Figure 1, further bacteriophage-enzymatic processing of the purified material, under precisely controlled conditions, leads to a reproducible partial digestion of this material. This resulted in the production of a homogeneous mono-disperse polysaccharide of average 900,000 molecular weight. This material was suitable for uniform thin-film formation (25 nanometers). Alternatively, complete limit digest of the material lead to high yield (64%) conversion of the native polymer to its basic repeat trimer. In this manner, gram quantities of oligosaccharide, with purity sufficient for crystallization, were produced.

Enzyme-bearing bacteriophage, which specifically infects *K. pneumoniae* K63 has been isolated and produced in large quantity. The bacteriophage's ability to specifically degrade K63 capsular polysaccharide has been used as a selection tool to generate a family of polysaccharides with related structures. Among the bacteria surviving this exposure to this bacteriophage are isolates which could be readily identified as having altered polysaccharide structure (see Table 1). These mutants showed a reduced level of non-stoichiometric pyruvylation of the capsular polysaccharide. Nuclear magnetic resonance (NMR) studies clearly identified mutants with changes in this structure of the polysaccharides repeat trimer.

As expected, the mutants with structurally altered polysaccharides showed altered rheological properties. In fact there was a significant improvement in both their drag-reducing effectiveness (over 10%) and mechanical, or shear stability (also above 10%). Furthermore, this material proved to be enzymatically convertible to oligosaccharides so that a new family of closely related structural oligomers has also been generated.

CONCLUSION

Bacterial polysaccharides represent a diverse and largely untapped source of polymeric materials and specialty chemicals, including possible starting materials for pharmaceuticals. The general area of adapting polysaccharides produced from bacterial sources is hampered economically by the high cost of purifying polysaccharides for commodity chemical uses and the limited ability to structurally modify native polymers to enhance performance[1].

Enzyme-bearing bacteriophages with endopolysaccharase activity have been demonstrated to be useful tools for polymer purification and processing. Furthermore, bacteriophage can be used as a selective agent to generate families of polysaccharides structurally related to native material, but with altered properties. In this way, new polysaccharides with enhanced properties can be generated with high probability. This report demonstrates the application of enzyme-bearing bacteriophage to select mutants of *K. pneumoniae* K63 with enhanced rheological performance.

We have identified the production of oligosaccharides for pharmaceutical biosynthesis as a possible important spin-off of our ongoing material science research. It should be clear that the bacteriophage enzyme techniques outlined here permit the generation of, not only new and novel, polysaccharides, but also large quantities of new oligosaccharides. Gram quantity production of these compounds in our laboratory has become routine.

Generation of oligosaccharides by the alternate route of bacteriophage enzymatic degradation of bacterial polymers, coupled with the ability to produce families of polysaccharide structural variants, provides a promising new source of materials for synthesis of biologically active carbohydrates.

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Enzymatic Processing of Polysaccharides Yields Material Suitable for Further Processing / Manipulation

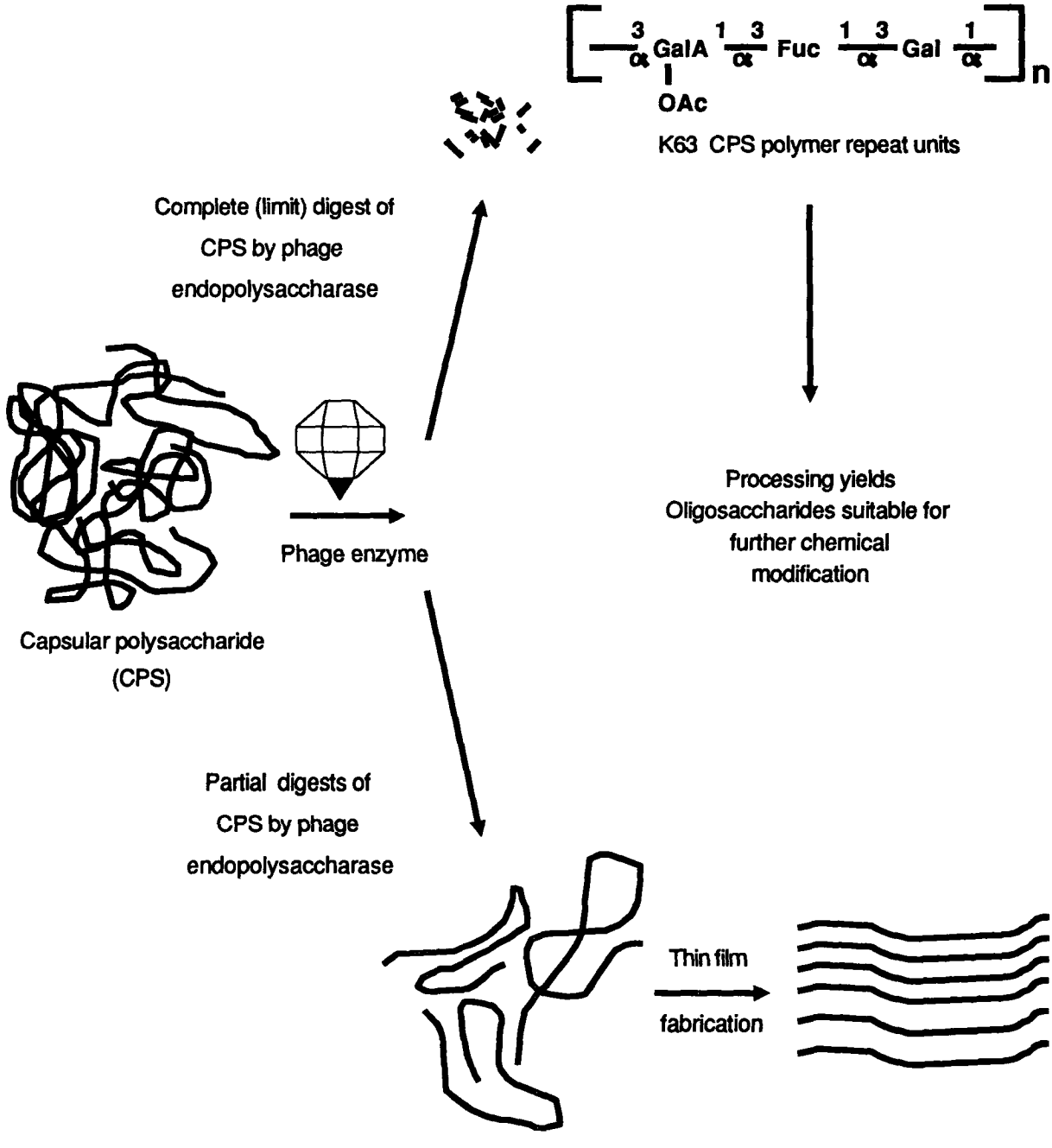

FIGURE 1

Table 1 Summary of Properties of *K. pneumoniae* K63 Variants

STRAIN	% PYRUVALATION[1]	NMR DONE = + Δ = difference	% DRAG REDUCTION[2]	% SHEAR STABILITY[3]
K63 wt	0.83	+	55.9	84.9
K63-JPL1	0.11	+ Δ (?)	67.7	95.6
K63-JPL2	0.04	+	66.7	95.6
K63-JPL3	0.11	+	67.0	99.4
K63-JPL4	0.12	+	67.6	98.2
K63-JPL5	0.11	+ Δ (GalA)	67.0	99.4
K63-JPL6	0.064	+	67.6	95.7
K63-JPL8	0.19	+	67.2	99.7
K63-JPL9	--	+ Δ (Fuc)	--	--

1. As % dry weight
2. % Drag-reduction at 25 wppm
3. % Drag-reduction of 25 wppm solution retained after three passes through rheometer